

The University of Chicago

STUDIES ON THE TOXINS AND ANTI-
TOXINS OF CLOSTRIDIUM
PERFRINGENS

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL
SCIENCES IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

1939

By

SARAH E. STEWART

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STUDIES ON THE TOXINS AND ANTITOXINS OF *CLOSTRIDIUM PERFRINGENS*¹

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Introduction

Titration of *Clostridium perfringens* toxins and antitoxins have yielded very inconsistent results in the hands of various workers. Such results are apparently dependent upon the interrelationship of the various antigenic components of the toxins that have been described. This suggests that the differences observed in the protective action of antitoxins may be due to the absence of certain antigenic components in the toxin used for immunization. A study has therefore been undertaken to determine some of the factors which influence the production of toxin with all the antigenic components for a given type, the nature of these antigens, and their significance in the production of an antitoxin which will give complete protection.

From theoretical consideration there were several possible factors that might be involved. (A) The nature of the toxin produced by a given strain might vary depending on: (1) Period of cultivation, (2) composition of the medium, (a) cysteine content, (b) meat and meat products in the medium, (c) glucose content. (B) If toxins vary in their composition depending on the medium used for cultivating the organisms the homologous antitoxins produced by immunizing with these toxins may vary depending on the presence or absence of the different components. (C) The nature of the different antigenic components may be such that the toxins are unstable.

Several different reactions are used for determining the titers of *perfringens* antitoxins. In 1920 Bengtson (1) promulgated a standard for the antitoxin of *Clostridium perfringens* of human origin. The unit was designated as that amount of antitoxin which would neutralize approximately 1,000 minimum lethal doses (m. l. d.) of a *perfringens* toxin. The tests were carried out by inoculating pigeons intramuscularly with different toxin-antitoxin mixtures. In 1930 the official unit for measuring the potency of the antitoxins was changed to one one-hundredth the former standard, as the unitage of antitoxins by the former method fell below 5 per cubic centimeter. This unit was adopted as the international standard (19).

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Other methods which are used for measuring the titers of *perfringens* antitoxin are: (1) *In vitro* hemolytic titrations (Henry (21), Mason and Glenny (25)); (2) intradermal necrotic tests (Glenny et al. (16)); and (3) the mouse protection test (10).

Using the above methods variations have been observed in the measurement of the protective power of certain antitoxins when tested against different lots of toxins. Marked irregularities have been reported with the method of *in vitro* hemolytic titrations (Henry (21), Weinberg and Guillaumie (49), Glenny (17), Prigge (33, 34)). Unsatisfactory results have also been reported with the mouse protection test (Weinberg and Guillaumie (49)).

At the National Institute of Health, up to 1934, the routine testing of *perfringens* antitoxins had been carried out by the intramuscular injection of pigeons. This method gave quite consistent results. In 1934 comparative tests of the mouse intravenous method and the pigeon intramuscular method were made (2). The results showed a very close correlation of these two methods with the particular toxins and antitoxins used. A short time later when the mouse intravenous method was applied to the routine testing of antitoxins very irregular results were obtained. Great variations in the potencies of certain antitoxins were observed depending on the toxins used. Similar variations were found when the American standard antitoxin was tested against different lots of toxins. Because of these irregularities a study was undertaken to determine the causes for such discrepancies.

Literature

Since *Clostridium perfringens* (*Bacillus aerogenes capsulatus*, *Clostridium welchii*) was first isolated by Welch and Nuttall in 1892 (50) the pathogenic action of the organism has been attributed to a variety of different products. At first the acid, principally butyric, produced in a culture was considered as the cause of the lesions of gas gangrene (8). The gas produced in the tissues was also believed to be of importance in the spread of the infection (27). In 1917 Bull and Pritchett (6) first demonstrated that *Cl. perfringens* produces an exo-toxin when grown under suitable conditions, and they showed that a specific antitoxin could be produced by immunizing animals to the toxin. The toxin has been shown to be actively hemolytic, the hemolysin and the toxin being considered as identical by Ouranoff (31), Ford and Williams (14), Wuth (55), and Mason and Glenny (25). Others, however, have observed a multiplicity of the toxin components. In 1920 Weinberg and Nasta (45) found that the proportion of hemolysin in toxins of different strains varied considerably. More recently (1930) Schnayerson and Samuels (35) in studying the blood changes produced by the hemotoxin of *Cl. perfringens* in pigeons described two hemotoxins which differed in the rapidity of their action. Henry (21), in 1923, suggested that the organism in addition

to the hemotoxin also produced a myotoxin. He was able to demonstrate a partial dissociation of the two lethal components by adsorption with ground fresh muscle. The muscle was found to reduce the toxicity though causing little change in the hemolytic activity. As the result of pathological studies, others have described nonhemolytic toxin components and designated them according to their specific action on certain tissues. Weinberg and Barotte (46) have described a neurotoxin which caused a degeneration of nervous tissue. Weinberg and Combiesco (47) have described a toxic factor which was shown to be specific for the walls of blood vessels.

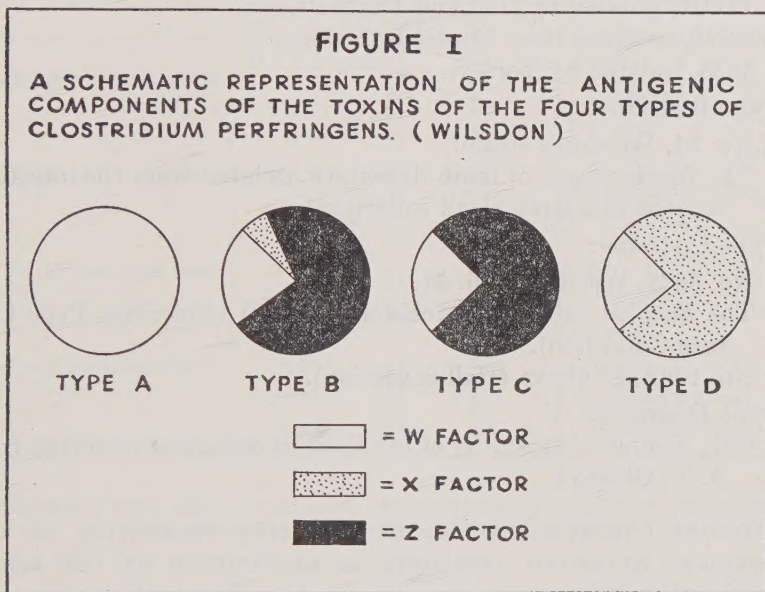
Still another toxic element attributed to this organism is the "non-specific toxin" described by Wassermann (42), Kojima (24), Kendall and Schmitt (23), and Walbum and Reymann (40). They found that this "toxin" was produced when *Cl. perfringens* was grown in a medium containing glucose. It was not neutralized by the antitoxin and was found to kill mice instantaneously when inoculated intravenously. Kendall and Schmitt (23) found this "nonspecific toxin" to be a histamine-like substance.

Prigge (33) has found that certain *perfringens* toxins contain two components which correspond to those described by Henry (21). He has been able to separate partially these two fractions by precipitating with sodium sulfate and ammonium sulfate (34). Prigge has proposed the term "zeta toxin" for the nonhemolytic factor and "alpha toxin" for the hemolytic factor. Previously the term "alpha" had been proposed by Glenn et al. (16) for the toxin produced by the *Cl. perfringens* strains of human origin. They believed that the toxin was a single antigenic entity which was hemolytic for red blood cells, lethal for mice, and necrotic following intradermal injections into guinea pigs or rabbits.

Until 1923 (15) only the toxins from strains isolated from human sources had been studied. With the investigation of strains isolated from animal sources the complexity of the toxic components became even more apparent. In 1928 Dalling (9) isolated the lamb dysentery bacillus which was found to be similar culturally and morphologically to Welch's organism. He showed, however, that his organism produced a highly potent toxin which was not neutralized by the antitoxin of the classical *perfringens* strains but that its antitoxin would neutralize the toxin of the human strains as well as its own toxin. About one year later, 1929-30, McEwen (26) isolated another *perfringens*-like organism which he named *Bacillus paludis*. This bacillus was isolated from sheep suffering from a disease of the enterotoxemia type called "struck." McEwen found that the toxin of his organism was not neutralized by the antitoxin of the *Cl. perfringens* strains of human origin nor would its antitoxin neutralize the toxins of these strains. Further investigations on the anaerobes concerned in diseases of sheep resulted in the isolation of a fourth *perfringens*-like

organism. Bennetts (3), in 1932, isolated an organism from sheep dying of an enterotoxemia in West Australia. This organism differed from the other known *perfringens* forms in its toxin, the toxin being neutralized only by its own specific antitoxin. Bennetts named this organism *Bacillus ovitoxicus*.

In 1931 Wilsdon (51) made a comparative study of the *perfringens*-like organisms and found that they could be classified according to their toxin-antitoxin reactions. He grouped them all as *Cl. perfringens* (*welchii*). Those of human origin, or the classical *Clostridium perfringens*, were designated as type A; the lamb dysentery organism, or *Bacillus agni*, as type B; *Bacillus paludis* strains as type C; and an



organism isolated by himself from an enterotoxemia of sheep as type D. Later he showed that his type D and Bennetts' *Bacillus ovitoxicus* were the same (52).

A schematic representation of the antigenic components of the toxins of the four types, as given by Wilsdon, is shown in figure 1.

The letter W represents the antigenic factor present in type A toxin. As this factor is present in the toxins of the other three types, the antitoxins of types B, C, and D are capable of neutralizing type A toxin, while type A antitoxin neutralizes only its own toxin. The type B toxin contains, in addition to the W factor, two other factors, Z and X, the Z factor being shared by type C toxin and the X factor by type D toxin. Because of the complexity of the B toxin its antitoxin is capable of neutralizing the toxins of all four types. The type C toxin containing only the two antigenic components Z and W is capable of inciting the production of an antitoxin which neutralizes only type A

and type C toxins. Since type D toxin also contains the W factor in addition to the X factor, its antitoxin is also capable of neutralizing type A toxin as well as its own. Wilsdon's work has been confirmed and extended by others (Glenny et al. (16), Borthwick (4), Weinberg and Guillaumie (48), and Duffett (13)).

Experimental

Cultures used.—The cultures used were as follows:

Type A strains:

PB6H, isolated by Bull and Pritchett.

SR12, received from Muriel Robertson.

1633, isolated by Torrey.

Type B strains:

No. 34, Wilsdon's strain.

The *Bacillus agni* of lamb dysentery, isolated from the intestinal content of a lamb (Hall collection).

Type C strains:

No. 3628, Wilsdon's No. 51.

The *Bacillus paludis* of McEwen (No. 40) (American Type Culture Collection).

No. 108A, as above (Hall collection).

Type D strains:

DR₂, Bennetts' strain R₂ of his *Bacillus ovitoxicus* (received from A. T. Glenny).

VARIATIONS OBSERVED IN THE NEUTRALIZING PROPERTIES OF THE AMERICAN STANDARD ANTITOXIN AS DETERMINED BY THE MOUSE PROTECTION TEST

By culturing under identical conditions, several lots of toxin were produced from three different strains of *Cl. perfringens* of human origin, PB6H, SR12, and 1633. These cultures were grown in glucose peptone beef infusion broth. Just before inoculation, the broth was heated in streaming steam for 1 hour to expel the air present, cooled rapidly, and then 0.25 percent glucose was added from a sterile 50 percent solution. Two-liter flasks of the broth were then inoculated with 6 cc. of the supernatant of a 24-hour meat culture. The broth cultures were then layered with sterile vaseline. These were incubated at 37° C. for 10 to 12 hours and then passed through Berkefeld N filters. The crude toxin filtrates were used in some of the tests; in others the toxins were precipitated with 70 percent ammonium sulfate and dried over phosphorus pentoxide. All the dried toxins used in this study were precipitated and dried in a similar manner.

The protection tests were carried out by the intravenous injection of mixtures of toxin and antitoxin into mice weighing 17 to 20 grams.

In order to determine the number of m. l. d. of each toxin neutralized, varying quantities of the toxin were added to a constant amount of antitoxin (0.2 unit). The mixtures were allowed to stand 1 hour at room temperature before being injected into animals.

A protocol of a representative test from one of a series is given in table 1.

TABLE 1.—*The neutralization of 3 different lots of type A Clostridium perfringens toxin by the American standard antitoxin*

Toxin	Amount of toxin	Amount of anti-toxin	Number of mice injected	Deaths	Time of death
	<i>M. l. d.</i>	<i>Unit</i>			
SR12 filtrate, 0.05 cc.=1 m. l. d..	4	0.2	4	3	10 minutes to 1 hour.
	7	.2	4	4	Immediately.
	10	.2	4	4	Do.
	13	.2	4	4	Do.
	16	.2	4	4	Do.
1633 filtrate, lot I, 0.05 cc.=1 m. l. d.	4	.2	4	1	15 minutes.
	7	.2	4	3	15 minutes to 11 hours.
	10	.2	4	4	Immediately.
	13	.2	4	4	Do.
	16	.2	4	4	Do.
1633, lot II pptd. and dried, 0.2 mg.=1 m. l. d.	8	.2	6	0	
	11	.2	6	0	
	17	.2	6	0	
	14	.2	6	0	
	20	.2	6	2	3 hours.
PB6H pptd. and dried, 0.6 mg =1 m. l. d.	2.5	.2	6	1	1 hour.
	5	.2	6	1	Do.
	7.5	.2	6	4	30 minutes to 1 hour.
	10	.2	6	6	5 to 15 minutes.
	12.5	.2	6	5	Immediately.

A. FACTORS WHICH MIGHT INFLUENCE THE NATURE OF THE TOXIN PRODUCED BY A GIVEN STRAIN OF *Cl. perfringens*, TYPE A

1. PERIOD OF INCUBATION

Tests were carried out to determine the effect of the period of incubation as a factor governing the production of a toxin component which is not neutralized by the antitoxin. Three 2-liter flasks of peptone beef infusion broth containing 0.25 percent glucose were heated and cooled as above and then each inoculated with 6 cc. of a 24-hour meat culture of strain SR12. These were incubated at 37° C. for 8, 11.5, and 20 hours. The cultures were then filtered and the toxins precipitated and dried as above. The m. l. d. of each toxin was determined in mice and subsequently tested against the standard antitoxin.

The three toxins were found to be neutralized equally well and with no irregularities. As many as 20 m. l. d. of each toxin were neutralized by 0.2 unit of the antitoxin. This is shown in table 2.

The differences observed in the neutralization of the toxin in this test and in the test shown in table 1 could not be explained on the basis of the data in this preliminary experiment. However, a partial explanation is presented in later experiments dealing with the

hemolysins. It may also be that certain of the toxin components vary in their stability and that precipitating with ammonium sulfate may alter the toxins.

TABLE 2.—*Period of incubation as a factor in the production of a toxin component not neutralized by the standard antitoxin*

SR12 precipitated toxin			Standard antitoxin	Number of mice inoculated	Deaths
Period of incubation	Percent glucose in medium	M. l. d. of toxin			
8 hours-----	0.25	8	Unit .2	3	0
		11	.2	3	0
		17	.2	3	0
		20	.2	3	1
11.5 hours-----	0.25	8	.2	3	0
		11	.2	3	0
		17	.2	3	0
		20	.2	3	2
20 hours-----	0.25	8	.2	3	0
		11	.2	3	0
		17	.2	3	0
		20	.2	3	2

2. COMPOSITION OF THE MEDIUM

(a) *Cysteine content*.—Orr and Reed (30) have shown that the addition of 0.1 percent or more of cysteine to chopped meat medium markedly inhibited the production of hemotoxin by *Cl. perfringens* and that hydrogen sulfide has a similar effect. They showed that the effect was related to the metabolism of the organism and not to a direct reaction with the formed toxin. The addition of these concentrations of cysteine was shown to produce a marked drop in the oxidation-reduction potential of cultures during the most active growth period. There were no observable differences in the rate of growth or of the maximum growth obtained. The presence of hemotoxin was determined by titrating against washed sheep red blood corpuscles. No mention is made of the toxicity of the cultures.

Since peptones of different makes differ in their sulphydryl content as determined by the sodium nitroprusside test, tests were carried out to determine the effect of different peptones, with and without added cysteine, on the hemolysins produced. Four different peptones were tested. These were used in amounts of one percent in beef infusion broth. Cysteine hydrochloride was added in amounts giving 0.015 percent and 0.15 percent concentrations. The media were tubed and sterilized by heating at 15 pounds pressure for 20 minutes. *Cl. perfringens* types A, B, C, and D were grown in the above media for 24 hours at 37° C. and the cultures were then centrifugalized. The supernatant fluid of each was then tested for hemolysins.

Hemolysin titrations of toxins produced in the different peptone media.—All titrations for hemolysins in this study were carried out identically. Five percent suspensions of washed sheep, rabbit, mouse, and human red blood cells were used in 0.5-cc. amounts. Varying quantities of toxin were added to the cells and these were then made up to a volume of 1 cc. with physiological saline. The toxin and red blood corpuscles were incubated for 1 hour at 37° C. and then read for the presence of hemolysis. The least amount of toxin producing complete hemolysis was designated as the minimum hemolyzing dose (m. h. d.).

The results on the hemolysin titrations of the type A toxins produced in the different peptone media are given in table 3.

The results on the hemolysins for the type C toxins were very similar to those of the type A toxins. Types B and D were only slightly hemolytic when grown in the media which gave good hemolysins with types A and C.

TABLE 3.—*The effect of the peptone and of the peptone plus cysteine on hemolysin production by Clostridium perfringens, types A and C*

Peptone	Amount of filtrate	Sheep r. b. c.		Rabbit r. b. c.		Mouse r. b. c.		Human r. b. c.	
		Type A	Type C	Type A	Type C	Type A	Type C	Type A	Type C
Bacto.....	Cc.								
	0.5	++++	++++	++++	++++	++++	++++	++++	++++
	0.2	++++	++++	++++	++++	++++	++++	++++	++++
	0.1	++++	++++	++++	++++	++++	++++	++++	++++
	0.05	++++	++++	++++	++++	++++	++++	++++	++++
Bacto+0.015 percent cysteine.	0.5	++++	++++	++++	++++	++++	++++	++++	++++
	0.2	++++	++++	++++	++++	++++	++++	++++	++++
	0.1	++++	++++	++++	++++	++++	++++	++++	++++
	0.05	++	++++	++++	++++	++++	++++	++++	++++
Bacto+0.15 percent cysteine.	0.5	++++	++++	++++	++++	++++	++++	++++	++++
	0.2	++++	++++	++++	++++	++++	++++	++++	++++
	0.1	++++	++++	++++	++++	++++	++++	++++	++++
	0.05	++	++++	++++	++++	+	+	+	+
Parke-Davis.....	0.5	++++	++++	++++	++++	++++	++++	++++	++++
	0.2	++++	++++	++++	++++	++++	++++	++++	++++
	0.1	++++	++++	++++	++++	++++	++++	++++	++++
	0.05	+	+	+	+	++++	++++	++++	++++
Parke-Davis+0.015 percent cysteine.	0.5	+++	++	+++	+++	+++	+++	+++	+++
	0.2	+++	++	+++	+++	+++	+++	+++	+++
	0.1	+++	++	+++	+++	+++	+++	+++	+++
	0.05	+	+	+	+	+	+	+	+
Parke-Davis+0.15 percent cysteine.	0.5	+++	++	+++	+++	+++	+++	+++	+++
	0.2	+++	++	+++	+++	+++	+++	+++	+++
	0.1	+++	++	+++	+++	+++	+++	+++	+++
	0.05	+	+	+	+	+	+	+	+
Proteose.....	0.5	+++	+++	+++	+++	+++	+++	+++	+++
	0.2	+++	+++	+++	+++	+++	+++	+++	+++
	0.1	+++	+++	+++	+++	+++	+++	+++	+++
	0.05	+	+	+	+	+	+	+	+
Proteose+0.015 percent cysteine.	0.5	++	++	++	++	++	++	++	++
	0.2	+	+	+	+	+	+	+	+
	0.1	—	—	—	—	—	—	—	—
	0.05	—	—	—	—	—	—	—	—
Proteose+0.15 percent cysteine.	0.5	+	+	+	+	+	+	+	+
	0.2	+	+	+	+	+	+	+	+
	0.1	—	—	—	—	—	—	—	—
	0.05	—	—	—	—	—	—	—	—

TABLE 3.—The effect of the peptone and of the peptone plus cysteine on hemolysin production by *Clostridium perfringens*, types A and C—Continued

Peptone	Amount of filtrate	Sheep r. b. c.		Rabbit r. b. c.		Mouse r. b. c.		Human r. b. c.	
		Type A	Type C	Type A	Type C	Type A	Type C	Type A	Type C
Witte.....	Cc.								
	0.5	++++	++++	++++	++++	++++	++++	++++	++++
	0.2	++++	++++	++++	++++	++++	++++	++++	++++
	0.1	++++	++++	++++	++++	++++	++++	++++	++++
	0.05	++++	++++	++++	++++	++++	++++	++++	++++
Witte+0.015 percent cysteine.	0.5	++++	++++	++++	++++	++++	++++	++++	++++
	0.2	++++	++++	++++	++++	++++	++++	++++	++++
	0.1	++++	++	++++	++++	++++	++++	++++	++++
	0.1	++++	++	++++	++++	++++	++++	++++	++++
	0.05	++	+	++	++	++	++	++	++
Witte+0.15 percent cysteine.	0.5	++++	++	++++	++++	++++	++++	++++	++++
	0.2	++++	++	++++	++++	++++	++++	++++	++++
	0.1	++	+	++	+	++	++	++	++
	0.1	++	+	++	+	++	++	++	++
	0.05	—	—	—	+	—	—	+	+

No marked differences in the hemolytic action of the toxins for the different types of red blood cells were observed. However, differences in the amount of hemolysin formed in the different media were noted. Cultures in the proteose peptone medium gave the poorest hemolysins; proteose peptone plus cysteine hydrochloride almost completely inhibited its production. Berkefeld filtrates of type A proteose cultures containing no hemolysin were tested for toxicity and found to be lethal for mice, indicating the presence of a nonhemolytic toxin component. The toxicity was comparatively low, the m. l. d. for a mouse being from 0.1 cc. to 0.5 cc. when inoculated intravenously.

(b) *Meat in the medium.*—The toxin of *Cl. perfringens* has been shown to be readily destroyed in an acid medium. Walbum and Reymann (40) have found that a potent toxin could be produced by culturing the organism in peptone veal broth if calcium carbonate were added to keep the pH from becoming too low. Meat has also been used in the medium because of its buffer effect (2).

In this study the meats that were used were ground beef heart, ground beef, and ground veal which had been extracted with water for the preparation of infusion broth. In order to use equivalent amounts of each, they were first thoroughly dried under a current of hot air. Each meat preparation was then tested for its buffer effect in the medium and for toxin production. The media were prepared by adding 10 percent of the dried meat to a peptone (any of the well-known brands) beef infusion broth and then adjusting the reaction so the pH after sterilizing would be about 7.8. The medium was sterilized by heating at 20 pounds pressure for 30 minutes.

Bacto beef, a commercial dried powdered meat preparation, was also used in broth. This was used in amounts of 2 to 2.5 percent. The media were prepared as above.

To study the toxin production, flasks of the above media were heated in streaming steam to expel the air and then cooled rapidly and 0.25 percent glucose from a sterile 50 percent solution was added. These were inoculated with a 24-hour culture of the strain under investigation. The cultures were incubated 16 to 20 hours at 37° C. After cultivation the pH was recorded, the cultures centrifugalized and filtered through Berkefeld N candles. The m. l. d. and hemolytic titration were then determined as above.

With all the above media very good toxins were produced with the strains used from the four types of *Cl. perfringens*. However, with type D culture 48 to 72 hours' incubation was required. The pH was in no instance below 6.0-6.2 after the period of cultivation. Only the specific results with the type A strains will be given as this is the chief toxin with which we are here concerned. It may be mentioned, however, that with the other three types very similar results were obtained.

Dried beef heart, beef, and veal in the medium all gave the same results so these will be grouped together as ground meat. This medium yielded toxins with an m. l. d. of 0.0125-0.025 cc. for a 17 to 20 gram mouse when inoculated intravenously. Such toxins were not markedly hemolytic. When tested against sheep red blood cells they were practically nonhemolytic if the readings were made after 1 hour incubation at 37° C. (0.5 cc. of the toxin filtrate giving about a 1 plus hemolysis). If the titrations were read after standing 24 hours at room temperature or in the refrigerator slightly more lysis was observed. Rabbit and human red blood cells were slightly more susceptible to the lysis of this toxin. However, when mouse red blood cells were used in the titrations, hemolysis was very pronounced. The hemolytic action of this toxin will be referred to its action on sheep red blood cells.

Toxins produced in the Bacto beef medium were also found to have high potencies. The m. l. d. of such toxin was often below 0.01 cc. for mice injected intravenously. These toxins were found to be highly hemolytic for all four types of red blood cells employed, mouse red blood cells being the least susceptible.

Decided differences in the rapidity of action of the two toxins have been observed. Toxins produced in meat medium and low in hemolysin for sheep red blood cells were found to be considerably slower in killing than toxins high in hemolysin for sheep red blood cells.

Table 4 gives the results of *in vitro* hemolytic titrations and of the lethal action of the two toxins produced by a single type A strain of *Cl. perfringens* when grown in the two media described above.

TABLE 4.—Titration of two type A toxins produced by the same culture in different media

Amount of toxin (cc.)	Hemolysin titration				Toxicity for mice	
	Sheep r. b. c.	Rabbit r. b. c.	Human r. b. c.	Mouse r. b. c.	Amount toxin (cc.)	Mice inocu- lated
Toxin filtrate from a type A peptone beef infusion broth culture containing 10 percent dried meat						
0.5.....	+	++++	++++	++++	0.5	Died.
0.2.....	+	+++	+++	+++	.2	Do.
0.1.....	+	++	++	++	.1	Do.
0.05.....	—	+	+	+	.05	Do.
0.025.....	—	±	—	±	.025	Do.
0.0125.....	—	—	—	+	.0125	Do.
0.006.....	—	—	—	+	.006	Lived.
Toxin filtrate from a type A Bacto beef peptone beef infusion broth culture						
0.1.....	++++	++++	++++	++++	0.5	Died.
0.04.....	++++	++++	++++	++++	.2	Do.
0.02.....	++++	++++	++++	++++	.1	Do.
0.01.....	++++	++++	++++	++++	.05	Do.
0.005.....	++++	+++	++	+	.025	Do.
0.0025.....	+++	+	+	±	.0125	Do.
0.001.....	+	—	—	—	.006	Lived.

The marked differences in the toxins produced in the Bacto beef medium and in the ground meat medium were found, as indicated below, to be due to the presence of lipid substances in the ground meat. Fifty grams of dried ground veal were finely pulverized and one-half was extracted with ether and alcohol, the method for extracting beef heart for the Kahn antigen (22) being used. The veal with the lipoids extracted was then dried and made up into medium using 10 percent of the defatted veal in peptone beef infusion broth, and the pH adjusted to 7.8. As a control the ground veal which had not been extracted with ether and alcohol was made up into medium in the same manner as the defatted veal. Types A and C *Cl. perfringens* were grown in both of these media for 16 hours and then filtered and the filtrate tested for hemolysins.

Both the type A and the type C grown in the defatted veal medium produced toxins with strong hemolysins for sheep, rabbit, and mouse red blood cells, while the filtrates of the cultures grown in the veal medium with the veal that had not been defatted were nonhemolytic for sheep red blood cells, only slightly hemolytic for rabbit red blood cells, but definitely hemolytic for mouse red blood cells. This is shown in table 5.

In order to determine whether the lipid substances inhibited hemolysin production, or merely masked the hemolysin present, or acted in some way to protect the sheep, rabbit, and human red blood cells, the supernatant fluids of centrifuged cultures grown in ground veal medium were thoroughly extracted with ether and tested

for hemolysins. The toxin remained nonhemolytic for sheep red blood cells and only slightly hemolytic for rabbit and human red blood cells. Reduction with sodium sulfite did not activate hemolysis. Hemolytic controls extracted in like manner remained hemolytic.

TABLE 5.—*The effect of lipoids on hemolysin production by Clostridium perfringens types A and C*

Medium	Amount of culture filtrate used, cc.	Sheep r. b. c.	Rabbit r. b. c.	Mouse r. b. c.
Type A toxin				
10 percent ground lean veal in beef infusion peptone broth....	0.5	+	++	++++
	.2	—	+	++++
	.1	—	—	++++
	.05	—	—	++++
	.025	—	—	+++
Same as above, only the ground veal was extracted with ether and alcohol.....	.1	++++	++++	++++
	.04	++++	++++	+++
	.02	++++	++++	+
	.01	++++	+++	—
	.005	+++	++	—
Type C toxin				
10 percent ground lean veal in beef infusion peptone broth....	0.5	+	++	++++
	.2	—	+	++++
	.1	—	±	++++
	.05	—	—	+++
	.025	—	—	+
Same as above, only ground veal was extracted with ether and alcohol.....	.1	++++	++++	++++
	.04	++++	++++	++++
	.02	++++	++++	+++
	.01	++++	+++	+
	.005	+++	++	—

From these results it appears that the lipoids either inhibit the formation of hemolysin for sheep red blood cells or in some way alter it.

(c) *Glucose in the medium.*—Several investigators have found that when *Cl. perfringens* is grown in a medium containing glucose a toxic substance which is not neutralized by the antitoxin is formed. Kojima (24) found that this "nonspecific toxin" was directly correlated with the percentage of glucose in the medium, being produced when the sugar content is high, the line of division being fairly constant at 0.5 percent glucose. Walbum and Reymann (40) found that it was not formed in measurable quantities in media containing 0.75 percent glucose. However, with a content of 2.25 percent glucose it was formed in considerable quantity.

In our work three type A strains, SR12, PB6H, and 1633, were grown in flasks of peptone beef infusion broth containing glucose in the following percentages: 0.25, 0.5, 1.0, 2.0, and 2.25. These were

incubated 12 hours, filtered through Berkefeld N candles, and the toxin filtrates tested for their m. l. d. Neutralization of these toxins was then determined by testing against two different antitoxins, one high in antihemolysin and the other low in antihemolysin as determined by titrations against toxins highly lytic for sheep red blood cells. (This will be shown in the section on the antigenic relationship of the hemolysins.)

As seen in table 6 all the toxins, even those produced in media containing a high percentage of glucose, were neutralized by antitoxin 229. With antitoxin AS₁ no protection was given against the toxins produced in the medium containing 1 or 2 percent glucose; as few as 2.5 m. l. d. were not neutralized. However, when this same antitoxin was tested against the toxins made in the media containing 0.25 and 0.5 percent glucose the mice were protected. Antitoxin AS₁, as contrasted with antitoxin 229, had very little antihemolysin for the lysis for sheep red blood cells.

TABLE 6.—The neutralization of the "nonspecific" toxin produced by *Cl. perfringens* (PB6H) when grown in a media containing different percentages of glucose

Toxin				Antitoxin			Toxicity for mice	Time of death (minutes)	Hemolytic action of toxin-antitoxin mixtures on red blood corpuscles			
Percent glucose	M. l. d. for sheep r.b.c. (cc.)	Amount (cc.)	Number m. l. d.	Number	Amount (cc.)	Dil.			Sheep	Mouæ	Rabbit	Human
0.25	0.02	0.05	2.5	229	0.2	1:2	Lived	---	---	---	---	---
		.125	5.0	229	.2	1:2	do	---	---	---	---	---
		.25	10.0	229	.2	1:2	do	---	---	---	---	---
.5	.04	.025	2.5	229	.2	1:2	do	---	---	---	---	---
		.05	5.0	229	.2	1:2	do	---	---	---	---	---
		.1	10.0	229	.2	1:2	do	---	---	---	---	---
1.0	.1	.25	2.5	229	.2	1:2	do	---	---	---	---	---
		.5	5.0	229	.2	1:2	do	---	---	---	---	---
		1.0	10.0	229	.2	1:2	Died	90	+++	+	+++	+++
2.0	.1	.25	2.5	229	.2	1:2	Lived	---	---	---	---	---
		.5	5.0	229	.2	1:2	do	---	---	---	---	---
		1.0	10.0	229	.2	1:2	do	---	+++	---	---	---
.25	.02	.05	2.5	AS ₁	.2	1:25	do	---	+++	---	---	---
		.125	5.0	AS ₁	.2	1:25	do	---	+++	---	---	---
		.25	10.0	AS ₁	.2	1:25	do	---	+++	---	---	---
.5	.04	.025	2.5	AS ₁	.2	1:25	do	---	+++	---	---	---
		.05	5.0	AS ₁	.2	1:25	do	---	+++	---	---	---
		.1	10.0	AS ₁	.2	1:25	do	---	+++	---	---	---
1.0	.1	.25	2.5	AS ₁	.2	1:25	Died	20	+++	+	+++	+++
		.5	5.0	AS ₁	.2	1:25	do	10	+++	+	+++	+++
		1.0	10.0	AS ₁	.2	1:25	do	1	+++	+	+++	+++
2.0	.1	.25	2.5	AS ₁	.2	1:25	do	40	+++	+	+++	+++
		.5	5.0	AS ₁	.2	1:25	do	30	+++	+	+++	+++
		1.0	10.0	AS ₁	.2	1:25	do	5	+++	+	+++	+++

Longer periods of incubation appear to have little effect on the production of a "nonspecific toxin." Cultures grown in meat medium or broth with added calcium carbonate, containing 2.25 percent glucose and incubated for 20, 24, 30, 36, and 41 hours, showed very little toxicity. In some instances 0.5 cc. was found to kill mice instantly when inoculated intravenously but the toxin was neutralized by antitoxin.

B. THE ANTIGENIC RELATIONSHIP OF THE HEMOLYSINS

In order to determine the toxicity and the antigenicity of the two hemolysins prepared in the different media, antitoxins were prepared against the two toxins. Rabbits were immunized against the toxins of types A, B, C, and D *Cl. perfringens*, using strongly hemolytic filtrates from peptone beef infusion broth cultures and Bacto beef cultures and with filtrates from cultures grown in ground meat medium which were nonhemolytic for sheep red blood cells.

Formalinized toxins (0.3 percent formaldehyde added and then incubated for 48 hours at 37° C.) were used for immunizing the rabbits. In order to assure good antihemolytic titers in the sera, when the rabbits had reached a fair degree of immunity, fresh toxin filtrates were used instead of the formalinized toxins. A total of 36 injections was given to each rabbit. Two rabbits were immunized against each toxin as follows:

Rabbit	Strain	Type	Medium
243	PB6H	A	Ground meat medium.
67	PB6H	A	Do.
229	PB6H	A	Peptone beef infusion broth and Bacto beef.
235	PB6H	A	Do.
164	108A	C	Ground meat medium.
244	108A	C	Do.
86	108A	C	Peptone beef infusion broth and Bacto beef.
241	108A	C	Do.
171	34	B	Ground meat medium.
181	34	B	Do.
18	34	B	Peptone beef infusion broth and Bacto beef.
16	34	B	Do.
226	DR ₂	D	Ground meat medium.
286	DR ₂	D	Do.
11	DR ₂	D	Peptone beef infusion broth and Bacto beef.
15	DR ₂	D	Do.

1. SPECIFICITY OF ANTICHEMOLYSINS

The antigenic relationship of the two hemolysins was determined by hemolytic titrations and by mouse intravenous inoculations of toxin-antitoxin mixtures. Each rabbit antiserum was tested against the two hemolysins produced by the homologous type. The hemolytic titrations and the protection tests were carried out as in the previous experiments. Two mice were used to each dose of toxin-antitoxin mixture. The size of the inoculum was kept to 1 cc. when possible.

It was found that antitoxins 235 and 229 which were prepared by immunizing with strongly hemolytic type A toxins gave complete protection against both hemotoxins of type A. Antitoxins 67 and 243 prepared by immunizing with the toxins that were not lytic for sheep red blood cells neutralized only their homologous toxins. The results are given in table 7.

TABLE 7.—*The antigenic relationship of the two types of hemolysins as determined by hemolytic titration with sheep red blood cells and by neutralization of the lethal effect*

Antitoxin number, 0.2 cc. of 1:10 dilution	Type A hemolytic toxin			Type A toxin, nonhemolytic for sheep r. b. c.		
	Amount toxin, m. l. d.	Hemolysin for sheep r. b. c.	Protection for mice	Amount toxin, m. l. d.	Hemolysin for sheep r. b. c.	Protection for mice
67.....	2	++	Lived.....	2	—	Lived.
	4	++++	Died.....	4	—	Do.
	8	++++	do.....	8	—	Do.
	16	++++	do.....	16	—	Do.
243.....	2	++++	do.....	2	—	Do.
	4	++++	do.....	4	—	Do.
	8	++++	do.....	8	—	Died.
	16	++++	do.....	16	—	Do.
235.....	2	—	Lived.....	2	—	Lived.
	4	—	do.....	4	—	Do.
	8	—	do.....	8	—	Do.
	16	—	do.....	16	—	Do.
	20	—	do.....	20	—	Do.
229.....	2	—	do.....	2	—	Do.
	4	—	do.....	4	—	Do.
	8	—	do.....	8	—	Do.
	16	—	do.....	16	—	Do.
	20	—	Died.....	20	—	Died.

The results were similar with types B, C, and D toxin-antitoxin mixtures. With types B, C, and D, as with type A, if the hemolysins produced in the broth cultures were not neutralized by the antitoxin, the toxin-antitoxin mixtures were toxic for mice when inoculated intravenously. The type B and D toxins of the strains used were never highly hemolytic for sheep red blood cells so the differences in the hemolytic and nonhemolytic toxins were not as pronounced as with the types A and C strains. These tests show the importance of producing antitoxins which have antihemolysins for the different types of hemolysins produced by *Cl. perfringens*.

2. HEMOLYSIN ABSORPTION AS FURTHER EVIDENCE OF THE SPECIFICITY

Toxin filtrates from ground meat cultures, broth cultures and Bacto beef cultures were absorbed with stroma from sheep, rabbit, and mouse red blood cells prepared according to the method of Pascucci (32). The absorption of the hemolysins was carried out by incubating 40 m. h. d. of the toxins with 50 milligrams of the different dried stroma. (The m. h. d. was determined with sheep red blood cells for the broth and Bacto beef toxins, while mouse red blood cells were used for the toxin produced in the ground meat medium.) The stroma and toxin were incubated overnight at 10° C., then one hour at 37° C. The toxins were then centrifugalized in order to remove the stroma and the clear supernatant tested for lysins present for sheep, rabbit, and mouse red blood cells.

The results of the hemolysin absorption tests of the three type A toxins are given in table 8.

TABLE 8.—*Absorption of the hemolysins of type A toxins with dried pulverized stroma of rabbit, mouse, and sheep red blood cells (40 m. h. d. of toxin + 50 mg. stroma)*

Medium used for producing the toxin	Red blood cell stroma	Hemolytic titrations		
		Sheep red blood cells	Rabbit red blood cells	Mouse red blood cells
Broth.....	Sheep.....	—	±	—
	Rabbit.....	—	±	+
	Mouse.....	—	±	+
Ground meat.....	Sheep.....	—	++	++++
	Rabbit.....	—	++	++++
	Mouse.....	—	++	++++
Bacto beef.....	Sheep.....	—	+++	++++
	Rabbit.....	—	+++	++++
	Mouse.....	—	+++	++++

The hemolysin present in broth culture filtrates was absorbed out by each of the three different types of red blood cell stroma used, the filtrate becoming nonhemolytic for all three types of cells. The absorption was nonspecific; that is, the lysin for sheep red blood cells was absorbed out by rabbit, mouse, and sheep red blood cell stroma. The same was true of the hemolysin for the rabbit and mouse red blood cells.

With the toxins prepared in the Bacto beef medium, which also had hemolysins for all three types of red blood cells, only the hemolysin for the sheep red blood cells was completely absorbed out, this absorption being nonspecific as all three types of red blood cell stroma removed it. The hemolysins for the mouse and the rabbit red blood cells were only partially absorbed.

The hemolysin of the toxin filtrates from cultures in ground meat medium differed from the hemolysin in the broth cultures in that it was not absorbed by red blood cell stroma. Only a very slight absorption was noted in testing for the hemolysin for rabbit red blood cells, while with mouse red blood cells no absorption appears to take place with any red blood cell stroma. The hemolysin was not removed even when the number of m. h. d.'s was decreased to 5 m. h. d. and the red blood cell stroma increased to 100 milligrams.

The same results were obtained with the hemolysins of types B, C, and D toxin filtrates.

C. THE NATURE OF THE TOXIN COMPONENTS OF *Clostridium perfringens* TYPE A

1. THE INACTIVATION OF THE HEMOLYSINS BY FILTRATION

Inactivation of hemolysins as a result of filtration through Seitz filters has been observed by Chopra and Roy (7). They explained the inactivation as due to the surface action of the asbestos which eliminates the active principle by adsorption or by altering its nature

altogether. A similar inactivation of the hemolysins of *Cl. perfringens* has been observed in our work as a result of filtering through collodion or cellophane membranes.

Filtration of the toxin of *Cl. perfringens* through graded membranes will be discussed in another communication.

Membranes through which hemolytic toxins had been filtered and which had completely removed the hemolysins from the filtrate were washed in a small volume of saline, about one-fourth that of the toxin filtered, and the washings tested for hemolysin. Although slight hemolytic activity could be demonstrated it was never of the original titer. Attempts to recover the hemolysins by grinding the membranes or by eluting, using buffers with a range from pH 5.0 to pH 9.4, were negative.

It was found that complete inactivation of the hemolysins on the membrane could be brought about by filtering a quantity of physiological saline through the membrane after filtering the toxin, thus washing it free of the broth present.

2. ACTIVATION OF THE HEMOLYSINS

The inactive hemolysins on the membrane could be recovered by washing the membrane in a small amount of physiological saline. This, as well as the first filtrate (the broth filtrate), was nonhemolytic. However, it was found that if the two were mixed, the mixture became hemolytic. This suggested the possibility of a cohemolysin in the filtrate. A similar activation of the hemolysins recovered from the membrane was produced by adding broth or 0.05 percent cysteine hydrochloride. The addition of two amino acids without SH groups, glycine and alanine, did not bring about an activation. It may be assumed, therefore, that the activation is due to a reduction of the hemolysins or that the hemolysins are capable of bringing about a lysis of red blood cells only when in a reduced medium. That the activation is due to a reduction either of the hemolysins or of the medium in which suspended can be further shown by bubbling oxygen through an active preparation. This procedure was found completely to inactivate the hemolysins, the reaction being reversible.

The activation of enzymes with reducing substances such as hydrogen cyanide, hydrogen sulfide, or organic substances containing sulphydryl groups in the reduced form has been extensively studied (18, 28, 39, 41, 43). Hellerman, Perkins, and Clark (20) believe that the phenomenon associated with the reversible inactivation of some enzymes is due to a direct action of the sulphydryl groups of the enzyme itself; the enzyme in its reduced form, for example En-SH, being active while the En-S-S-En oxidized form is inactive.

A reversible inactivation of hemolysins similar to that described for enzymes has also been shown. Neill (29) found that pneumococcal

hemolysin which had been inactivated by air or hydrogen peroxide may be reactivated by the use of reducing substances. Shwachman, Hellerman, and Cohen (37) showed that the pneumococcal hemolysin is active when reduced and may be reversibly inactivated by operations that are known to convert sulfhydryl compounds to the corresponding dithio derivatives or to mercaptides.

If an enzyme or a hemolysin possesses SH groups which determine its activity the active form of a purified enzyme or hemolysin might be expected to give a positive nitroprusside test and the inactive form to become activated on the addition of hydrogen cyanide.

In testing the effect of hydrogen cyanide on *perfringens* hemolysin it was found that at pH 7.0 concentrations of 0.025, 0.05, and 0.1 percent had no effect on the activation of the hemolysin inactivated by filtration. Concentrations of 0.1 and 0.05 percent were found to be inhibitory to the action of active hemolysin but 0.025 percent had no effect. Hydrogen cyanide has been shown to have an inhibitory effect on certain enzymes (36, 38). Results of the activation of *perfringens* hemolysin are given in table 9.

TABLE 9.—*The action of activating substances on the hemolysin inactivated by filtering through a cellophane membrane*

Hemolysin	Dilution	Amount, cc.	Hemolytic activity		
			Sheep r. b. c.	Rabbit r. b. c.	Mouse r. b. c.
Hemolysin separated from broth by filtration, recovered with saline.	Undiluted-----	.5	—	—	—
		.2	—	—	—
		.1	—	—	—
		.05	—	—	—
Above+nonhemolytic filtrate-----	1:1-----	.5	++++	++++	++++
		.2	++++	++++	++++
		.1	++++	++++	++++
		.05	++	++	+
Above hemolysin+broth-----	1:1-----	.5	++++	++++	++++
		.2	++++	++++	++++
		.1	++++	++++	++++
		.05	++	++	+
Above hemolysin+0.05 percent cysteine hydrochloride.	Undiluted-----	.5	++++	++++	++++
		.25	++++	++++	++++
		.1	++++	++++	++++
		.05	++	++	+
Above hemolysin+0.05 percent KCN +0.025 percent KCN.	-----do-----	.5	—	—	—
		.5	—	—	—
Original active hemolysin+0.05 percent KCN+0.025 percent KCN.	-----do-----	.5	—	—	—
		.5	++++	++++	++++

3. A NONHEMOLYTIC TOXIN COMPONENT

At times the filtration of *Cl. perfringens* type A toxins through colodion membranes resulted in the separation of a toxin component in the filtrate which was nonhemolytic for rabbit, mouse, and sheep red blood cells as determined by *in vitro* titrations. This toxin was lethal

for mice, the m. l. d. being from 0.1 cc. to 0.5 cc. It was impossible to obtain consistent results. The production and filtration of the non-hemolytic component is dependent upon factors not yet determined.

Discussion

The variations observed in the neutralizing properties of certain *Cl. perfringens* type A antitoxins when tested against different lots of type A toxins appear to be due to differences in the composition of the toxins. The nature of the toxin produced by a given strain has been shown to be dependent on the medium.

Recently Dalling and Ross (12), in studying the factors influencing the production of the various toxins of the *Cl. perfringens* group, have shown the importance of meat in the production of the epsilon factor (Glenny et al.) or Wilsdon's X factor by types D and B cultures. They found that an epsilon toxin of high value could be obtained by culturing in a medium with 50 percent meat by volume and that with type B cultures it was practically negligible in the absence of meat.

Since the medium used in culturing the *Cl. perfringens* group determines to a large extent the toxin components formed, the stability of the types is also more or less influenced by the medium. Dalling (11) has reported a type B which became permanently altered so that it lost its capacity to produce the epsilon factor, and so became toxicogenically a type C. Dalling and Ross (12) have reported a type C which at times failed to produce any beta toxin (Glenny et al.). It differed from the type A cultures in that it produced a delta hemolysin (Glenny et al.) as well as the alpha toxin. Borthwick (5) has described the conversion of a type D to a type A.

A résumé of the toxin components which have been described for the toxins of the *Cl. perfringens* group is as follows:

Type A.—Alpha toxin (Glenny et al.) or the W factor (Wilsdon); this is hemolytic, lethal, and necrotic; and a zeta (Prigge) or nonhemolytic toxin factor.

Type B.—Alpha toxin; beta toxin (Glenny et al.), or the Z factor (Wilsdon); this is lethal and necrotic; a gamma toxin (Glenny et al.) and an epsilon toxin (Glenny et al.), or the X factor (Wilsdon). The gamma and epsilon toxins are also lethal and necrotic.

Type C.—Alpha, beta, gamma, and a delta toxin (Glenny et al.). The delta fraction is a hemolysin and is believed to be nontoxic.

Type D.—Alpha and epsilon factors.

In this study, although many of the details remain to be worked out, our results show that the toxin of *Cl. perfringens* type A when produced under suitable conditions is a complex substance made up of at least three components, two hemotoxins and a nonhemolytic toxin. The production of *Cl. perfringens* type A antitoxins with antibodies for all the toxin components depends on the use of a toxin pro-

duced in a medium which yields all the toxin components. A medium made with 10 percent dried defatted ground meat or 2.0 to 2.5 percent Bacto beef in a peptone beef infusion broth has been found suitable for this purpose.

The factors which influence the production of the nonhemolytic toxin have not been determined. Its presence as determined by filtration through collodion and cellophane membranes was quite irregular. This irregularity of the nonhemolytic toxin in the filtrates may have been due to differences in the physical properties of the membranes; some may have retained it along with the hemolysins while others allowed it to pass; or it may be that the nonhemolytic toxin is formed only after a definite period of growth and is unstable. Nonhemolytic filtrates which were toxic could not be made hemolytic by reducing, using the usual methods of reduction. The two other toxin components, the hemotoxins, may be reversibly inactivated. The lytic activity of the hemolysins appears to be controlled by the state of oxidation of either the lysins themselves or of the medium in which they are suspended. When the hemolysins were completely separated from the broth in which they were suspended they lost all their lytic activity. On the addition of the nonhemolytic filtrate to the inactivated hemolysins the lytic property of the hemolysins returned. Broth and cysteine hydrochloride were found to have the same activating effect. The activation of the hemolysin may have been due to one of the following reactions: (1) The addition of sulfhydryl groups to the hemolysin, (2) a reduction of the sulfhydryl groups in the hemolysin, (3) a reduction of the medium in which the hemolysin was suspended. It was not determined which reaction took place. It was shown, however, that potassium cyanide added to the inactive hemolysin did not activate it or give a positive nitroprusside test showing that the S-S groups were changed to SH groups. It may be added, however, that this test has been reported as not altogether reliable in testing for the thiol group (37).

The hemolysin that is lytic for sheep red blood cells seems to be the less stable of the two hemotoxins. Under certain conditions precipitating with ammonium sulfate appears to inactivate the hemolysin to a marked degree.

The "nonspecific" toxin resulting from the cultivation of *Cl. perfringens* type A in media containing from 1 to 2.25 percent glucose was found to be neutralized by antitoxins which had high anti-hemolytic titers.

Summary

Cl. perfringens type A has been shown to produce at least three different toxin components which are dependent on the type of medium used for culturing the organism. Two of the components are hemotoxins which can be separated by absorption on dried red blood cell

stroma and by filtration. One is lytic for sheep red blood cells while the other has little action on them after 1 hour's incubation at 37° C. These two hemotoxins were shown to be antigenically different. Lipoids were found to inhibit the formation of the hemotoxin which is lytic for sheep red blood cells.

The hemotoxins may be reversibly inactivated by separating them from the broth in which they are suspended or by bubbling oxygen through the active hemolysins. The hemotoxins may be separated from the broth by filtering through collodion membranes. Activation of the hemolysins can be brought about by introducing substances containing SH groups.

A nonhemolytic toxin component was found in some of the type A filtrates. It was possible to separate this toxin from the hemolysins by filtering through graded membranes.

The "nonspecific toxin" resulting from the cultivation of *Cl. perfringens* type A in media containing from 1 to 2.25 percent glucose was found to be neutralized by antitoxins having high antihemolytic titers for the two types of hemolysins.

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